# TRACKING FLORAL VISITATION USING DNA BARCODES

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Columbus State University

The College of Science

The Graduate Program in Environmental Science

# Tracking Floral Visitation Using DNA Barcodes

A Thesis in

Environmental Science

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

December 2010

I have submitted this thesis in partial fulfillment of the requirements for the degree of Master of Science.

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## ABSTRACT

Floral visitation resulting in interspecific pollen transfer (IPT) from non-native or invasive plant species can affect the reproductive fitness of native plant species through pollen allelopathy, stigma clogging, stylar clogging, and ovule (seed) discounting. The prevalence of IPT and the importance of pollinators in mediating its impacts, however, remain poorly understood. Although most traditional methods for examining visitor movement are insufficient for determining rates of potential IPT, one promising alternative is the use of DNA barcoding. Because floral visitors eat, collect or unknowingly obtain pollen, nectar and other floral tissues, plant DNA should be recoverable from their bodies, permitting molecular identification of pollen loads. To assess the utility of plant DNA barcodes for tracking floral visitation, I collected 89 honeybees (*Apis mellifera*) and 49 bumblebees (*Bombus* spp.) from a disturbed forest edge in Columbus, Georgia and analyzed their pollen loads using the plastid DNA barcode region *rbcL*. The identities of monospecific pollen loads were determined by comparing sequences to a local plant reference library containing 22 native and 27 non-native plant species. The overall recovery of *rbcL* sequences from pollen loads was relatively high (41.6%). Based on local BLASTn analysis, 95% of monospecific pollen loads were identifiable to the species level. Rates of heterospecific visitation were quite high (77%), as indicated by pure heterospecific (15%) and mixed pollen loads (62%). Collectively, these results indicate that 1) plant DNA barcode sequences can be recovered from pollen loads; 2) species-level identification of pollen loads can be determined with high accuracy; and 3) levels of heterospecific visitation and potential IPT can be assessed

with DNA barcoding. As a new means of rapidly and effectively detecting potential pollen transfer between plant species that share floral visitors, my study demonstrates that DNA barcode analysis of pollen loads will contribute greatly to the rapidly growing field of ecological barcoding.

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ACKNOWLEDGEMENTS

I would first like to thank Dr. Bob Boyd, Dr. Kevin Fielman and Dr. Scott Santos from Auburn University for encouraging me to be innovative in my research and for helping me to develop the original ideas that led to the formation of this project.

I would like to thank Dr. Kevin Burgess for taking me on as a student with so little notice and with so much already on his hands. Thank you for allowing me the opportunity to contribute to this awesome field of research and for working so hard to make sure that financial support was available to complete this work.

To Dr. John Barone and Dr. Julie Ballenger, thank you for your time and constructive criticism while serving as my thesis committee members. I would like to express my gratitude to Dr. Roger Brown and Dr. Schwartz for their help in creating and photographing pollen slides. Also, thank you to so many other faculty members for providing books, equipment and overall support.

I am grateful to Dr. Bill Frazier and the Environmental Science Department of Columbus State University for providing me the opportunity to present my research at international meetings for the past two years.

Last, but certainly not least, I would like to thank Katharine Korunes, who probably deserves a thesis of her own for her contributions to this project. Without your countless hours of hard work and companionship in the field and lab, this research would have been impossible. Thank you for being so dedicated and for not running in the other direction when I told you we would be catching bees with tweezers.

## INTRODUCTION

The consequences of direct interactions by non-native or invasive plants with native plant species have been well-studied. In general, invasive species are considered superior competitors and may impact populations of native plant species through competition for nutrients, water, light, and space (Brown *et al.* 2002). Little is known, however, about the indirect effects of non-natives, such as their impact on the pollination and reproduction of native plant species (Brown *et al.* 2002, Bartomeus *et al.* 2008). It is now recognized that non-native plants have the ability to affect native plant species through shared pollinators (Morales & Traveset 2009) and that the presence of non-native species can reduce the reproductive fitness of native species through alterations in visitation rates (Tscheulin *et al.* 2009), as well as through changes in the composition of pollen carried by floral visitors (Brown & Mitchell 2001, Nielsen *et al.* 2008, Kandori *et al.* 2009).

When a pollinator or visitor forages on multiple plant species, pollen from a heterospecific plant species may be deposited on a plant's stigma, resulting in interspecific pollen transfer, or IPT (Waser 1978). IPT between closely related species can result in gene flow and has the potential to significantly impact the evolutionary trajectories of species and populations (Brown & Mitchell 2001). Some of the genetic consequences that may occur include the reduction of adaptation to local conditions, heterosis, or even local extinction by genetic assimilation (Ellstrand 1992). IPT can also affect the reproductive fitness of native plant species through pollen allelopathy, stigma clogging, stylar clogging, and ovule (seed) discounting (Brown *et al.* 2002, Kandori *et al.* 2009). In addition to the deposition of foreign pollen, IPT is often associated with a

decrease in the deposition of conspecific pollen, or conspecific pollen loss, which can lead to pollen limitation and reduced seed set (Morales & Traveset 2009). The prevalence of IPT and the importance of pollinators in mediating its impacts, however, remain poorly understood (Brown *et al.* 2002, Bartomeus *et al.* 2008).

Because most plants are animal-pollinated (National Research Council 2007), it is inevitable that floral visitors play an important role in determining the extent to which plant species are affected by the consequences of IPT. Thus, an examination of visitor pollen loads can be used to estimate IPT and its associated consequences at the level of flowers, individuals, or even species (Bartomeus *et al.* 2008). Researchers, however, have often failed to use direct examination of pollen loads when exploring pollination mutualisms (Alarcon 2009). Instead, most studies have tracked individual insects or used stationary observation of visits to individual plants. Floral visitors, though, are often difficult or impossible to follow (Valentini *et al.* 2009), and the observation of floral visitation can be time consuming and impractical (Memmott 1999), especially in species with infrequent visitation or rare visitors (Widmer *et al.* 2000). Indirectly, researchers have also used dye powders (Brown & Mitchell 2001), histochemical stains (Peakall 1989), and magnetic tags (Gary *et al.* 1971) to study pollinator movement, but these methods are time-consuming and impractical for large-scale investigations. Consequently, even direct examination of pollen loads through the identification of pollen or pollinia has limited value for determining the details of interactions, as pollen identification based on morphological characteristics is both difficult and imprecise (Zhou *et al.* 2007). For investigating the effects of non-native species, direct examination of pollen loads is also especially likely to be problematic because the impact of non-

native plants has been correlated with phylogenetic relatedness (Morales & Traveset 2009), and closely related species are more likely to possess highly similar pollen morphology.

One promising alternative in studying pollinator visitation is the use of DNA barcoding (Hebert *et al.* 2003, Janzen *et al.* 2009), a technique which uses a short standardized genetic marker, or group of markers, to provide rapid DNA-based identification of organisms (Borisenko *et al.* 2009, Plant Working Group CBOL 2009). The mitochondrial *CoI* barcode region has been useful for discriminating animal species and has already proven effective for documenting biodiversity (Hebert *et al.* 2004, Milankov *et al.* 2008) and in conservation efforts (Stahls *et al.* 2009, Weese & Santos 2009). For plants, the plastid markers *rbcL* and *mat*K have proven more effective (Plant Working Group CBOL 2009). To date, researchers have used putative DNA barcodes to determine animal diets from gut contents of both predators (Agusti *et al.* 2003, Barnett *et al.* 2010, Dunn *et al.* 2010) and herbivores (Matheson *et al.* 2008, Navarro *et al.* 2010), as well as from feces (Bradley *et al.* 2007). Because floral visitors eat, collect or unknowingly obtain pollen, nectar and other floral tissues (Davis 1996), plant DNA should be recoverable from their bodies, permitting similar identification of pollen loads through the use of plant DNA barcodes.

Considering current unprecedented rates of alien plant invasions (Mooney & Cleland 2001) and global reports of pollinator declines (NRC 2007), a new means of rapidly and effectively inspecting plant-pollinator interactions is urgently needed. As such, the overall goal of my thesis is to explore a novel use of plant DNA barcodes in order to address several questions related to IPT: 1) Can DNA barcode sequences be

recovered from pollen loads?; 2) Can the composition of pollen loads found on floral visitors be determined to the species level?; and 3) What proportion of those pollen loads indicate heterospecific visitation and the potential to contribute to IPT? To address these questions, I collected honeybees (*Apis mellifera*) and bumblebees (*Bombus* spp.) from a local invaded habitat and analyzed their pollen loads using the plastid DNA barcode region *rbcL*.

## MATERIALS AND METHODS

### Study Site

During May 2009, plants and insect visitors were sampled from a disturbed forest edge habitat on the Columbus State University campus, Columbus, Georgia (N 32.4996° W 84.9367°). The site contained a moderate diversity of flowering plant species typical of the Piedmont and Coastal Plain floras (Radford *et al.* 1968, Weakley 2008, USDA 2009), as well as an abundance of pollinators, including honeybees and bumblebees. Due to the site's disturbed nature, several invasive plant species had become established. The site also included a number of groups (Asteraceae, *Oenothera*, *Oxalis*, Verbenaceae) with closely related species, sharing similar pollen morphologies, which would present a considerable challenge for the determination of pollen species by traditional methods.

### Sampling

#### *Plants*

Vouchers were collected for all plant species in flower (excluding grasses) within the

study area, as well as within a 100 m radius of the boundary of the study area. All specimens were identified to the lowest taxonomic level (Radford *et al.* 1968, Weakley 2008, USDA 2009), mounted on herbarium sheets, photographed, and stored at the Columbus State University Herbarium (COLG) as barcode vouchers (Appendix A). To establish a pollen reference collection for microscopy analysis (see Methods, *Microscopy*), pollen samples were also collected from each plant species in flower. In order to generate a local plant barcode library, 3-5 $cm^2$ of leaf tissue was collected from each species and stored at -20°C until DNA extraction (see Methods, *DNA extraction*).

*Insect visitors and pollen loads*

Insect collections were made during four weekly collection periods from May 11 to June 1, 2009, between 10 A.M. and 4 P.M. During these bouts, all insect floral visitors were collected. Visitation was defined as physical contact of an insect with a flower or inflorescence combined with an associated observation of plausible foraging behavior, such as probing flowers with mouthparts, nectar robbing, or gathering pollen. Insects were collected directly from the flowers using tweezers in order to avoid contamination and once captured, were individually placed in 1.5 mL microcentrifuge tubes, stored on ice, and then frozen at -20°C.

Insects were thawed, and pollen loads were washed from each specimen by vortexing and inverting insects in Millipore (Millipore, Bedford, MA, www.millipore.com) filtered water for 30 s, or until a considerable proportion of the pollen was removed. Most insects were washed with 1.0 mL $H_2O$ in 1.5 mL microcentrifuge tubes, but larger specimens, such as *Bombus* and *Xylocopa* spp., were

washed with 2.0 mL $H_2O$ in 15 mL centrifuge tubes. Insects were then removed from the tubes, and the pollen load solutions were centrifuged at 17,500 rpm for 2 min. The pollen pellet and ~100 µl of the supernatant were retained for further analysis.

All insects were identified to family using Mitchell (1960); however, only honeybees and bumblebees were chosen for further analysis. The benefits of selecting these taxa included their overall abundance at the site, agricultural importance, and major relevance to current literature, including documented pollinator declines (National Research Council 2007). These individuals were identified to the species level using an online key (Ascher *et al.*2008), pinned, and deposited at the Columbus State University Invertebrate Museum.

Pollen analysis

*Microscopy*

Reference pollen material and pollen loads were examined via microscopy in order to substantiate molecular analysis. For pollen reference material, whole flowers or anthers collected from blooming plant species were vortexed in ~1.0 mL of Millipore water in 1.5 mL microcentrifuge tubes to release pollen. Non-pollen plant debris was removed with tweezers, and the solutions were centrifuged at 17,500 rpm for 2 min. The supernatant was removed in order to eliminate any residual debris. For pollen load microscopy, the pollen pellets created by centrifuging (see Methods, *Insect Visitors and Pollen Loads*) were re-suspended in the remaining supernatant, and ~30 µL of the resulting pollen load solutions were processed by methods modified from Kearns &

Inouye (1993). Both pollen reference and pollen load solutions were diluted to 1.0 mL in their 1.5 mL microcentrifuge tubes, and the pollen was dyed via the addition of ~10 µL fuchsin red stain. After 5 min, the solutions were centrifuged 1 min at 17,400 rpm. The supernatant was removed, 1.0 mL 100% ethanol was added, and the solutions were vortexed to eliminate any clumping. The pollen was allowed to settle and was then removed using a pipette and mixed into glycerin jelly on heated slides. The mixtures were covered with slide covers and were sealed with nail polish after cooling. The pollen reference slides were then viewed under compound microscopes and used to create a local pollen atlas. Online sources were used to provide pollen morphologies for any taxa not represented by a pollen reference slide (Davis 2001). Pollen load slides were analyzed in order to determine whether bees carried conspecific, heterospecific or mixed pollen.

*DNA extraction*

In order to generate a local plant reference library, ~100 mg frozen or ~200 mg dry leaf tissue from each plant species was pulverized via FastPrep®-24 (MP Biomedicals, Solon, OH, USA, www.mpbio.com), and DNA was extracted using the DNEasy Plant Mini Kit (Qiagen, Valencia, California, USA, www.qiagen.com) or the FastDNA® Kit (MP Biomedicals), according to the manufacturers' instructions.

DNA was also isolated from honeybee and bumblebee pollen loads for pollen load analysis. In total, 125 pollen loads were selected from female bees, which were collected from six plant species that received visitation by both visitor types. Those plant species were *Hieracium pilosselloides* (Asteraceae), *Oxalis rubra* (Oxalidaceae), *Stachys floridana* (Lamiaceae), *Triadica sebifera* (Euphorbiaceae), *Trifolium repens* (Fabaceae),

and *Verbena brasiliensis* (Verbenaceae). For DNA isolation, the pollen pellets created by centrifuging (see Methods, *Insect Visitors and Pollen Loads*) were re-suspended in the remaining ~100 µL supernatant by pipetting, and 70 µL of each of the resulting solutions was processed using the FastDNA® Kit, following manufacturer's instructions, except for the addition of a second wash step using reagents supplied with the kit.

*Amplification and sequencing*

Polymerase chain reaction (PCR) amplification of a 607 bp region of *rbcL* was performed for all successfully isolated plant and pollen load DNA. The *rbcL* primers (rbcL F and rbcLajf634R) and general PCR conditions were modified from Fazekas *et al.* (2008). PCR amplification was performed in 20 µL reactions, each containing 2 µL genomic DNA template (~30 ng), 0.8 U Ampli*Taq* Gold Polymerase with GeneAmp 10X PCR Buffer II (100 mM Tris-HCl pH 8.3, 500 mM KCl) and 2.5 mM MgCl2 (Applied Biosystems, Foster City, CA, www.appliedbiosystems.com), 0.2 mM dNTPs, 0.1 mM of each primer, and 5% D-(+)-Trehalose. Following an initial step of 5 min at 95 °C for enzyme activation and template denaturation, the PCR was performed with a touchdown amplification program of ten touchdown cycles from 58 to 53.5°C (1 min at 95°C, 40 s at 58-53.5°C and 1 min at 72°C), 30 additional cycles of 1 min at 95°C, 40 s at 54°C, 1 min at 72°C, and a final extension period of 5 min at 72°C. PCR was also performed on known ratios of *Oxalis rubra* and *Stachys floridana* DNA mixtures to provide a reference for interpreting heterozygous chromatographs produced by mixed pollen loads (Fig. 1). PCR amplification products were submitted to Functional Biosciences, Inc. (www.functionalbio.com) to be purified using ExoSAP (Exonuclease I, Shrimp Alkaline

Phosphatase) and sequenced in both directions with the primers used for amplification. All plant sequences were then submitted to the Barcode of Life Data (BOLD) Systems (www.boldsystems.org) and GenBank (www.ncbi.nlm.nih.gov).

*Sequence Analysis*

Sequence chromatograms were edited and assembled using CodonCode Aligner version 3.0.3 (CodonCode Corporation, Dedham, MA, www.codoncode.com) and aligned manually in Se-Al version 2.04 (http://tree.bio.ed.ac.uk/software/seal/). By comparison to heterozygous reference sequences (Fig. 1) and the surrounding sequence quality, putative heterozygous positions were identified and scored as ambiguous bases. Sequences with fewer than ten ambiguous positions were considered homozygous, indicating a pollen load composed of a single pollen type. This single (homospecific) pollen type could then be identified as a con- or heterospecific pollen load (see below). Sequences with ten or greater were considered heterozygous (see Yuan *et al.* 2004 for a similar approach), indicating mixed pollen loads. These pollen loads were interpreted as heterospecific because they, by nature, contained at least one foreign species of pollen.

In order to determine the source of homospecific pollen loads, a local plant sequence library was constructed in Geneious Pro 4.8.5 (Drummond et al. 2009), based on *rbcL* sequences obtained from plant species flowering at the site. Homozygous pollen load sequences were then compared to the local plant sequence library using BLASTn for short nearly exact searches (v. 2.2.22 as a plugin in Genious Pro 4.8.5). Species assignments were made by selecting sequence matches with the greatest % identical sites. By comparing each pollen load's species assignment to each visitor's collection

information, pollen loads could be identified as conspecific or heterospecific, relative to the plant species on which it was collected.

Heterozygous (or mixed pollen load) sequences were further analyzed to assess the validity of heterozygous peaks. This was accomplished by evaluating the proportion of ambiguous bases occurring at informative sites. Informative sites were identified within the plant reference sequences, and a consensus sequence was created, which represented all of the collected plant species. Each heterozygous sequence was then compared to the consensus sequence to determine the number and relative percentage of ambiguous bases occurring at nucleotide positions informative among plant species found at the study site.

*Statistical analysis*

A Chi-squared contingency test was used to compare sequence recovery rates of honeybees and bumblebees. One-way analysis of variance (ANOVA) was used to compare proportions of heterospecific visitation by honeybees and bumblebees, as well as to introduced and native plant species. Two-way ANOVA was used to test for the interaction between plant nativity and visitor type.

## RESULTS

### Sampling

*Plant reference library*

Forty-nine plant taxa were collected, representing 24 families and 45 genera (Appendix A). Over half of these (55.1%) were non-native, and three species were designated as

noxious weeds by the U.S. Forest Service (2010). The *rbcL* plant barcode region was sequenced for all specimens collected. Full length (607bp), bi-directional sequences were obtained for 47 (95.9%). An all-to-all BLAST within Geneious Pro 4.8.5 revealed that 47 of the 49 plant taxa (95.9%) possessed unique *rbcL* sequences, allowing them to be easily discriminated from one another. These reference sequences are available on the BOLD Systems website and GenBank (Appendix A). Pollen reference slides were completed for 35 (71.4%) of the taxa, and online data were retrieved for the rest.

*Insects and pollen loads*

A total of 389 visitors from three insect orders were acquired over 33 cumulative hours of collection across the four sampling periods (Table 1). Most of the visitors were bees (71.8%). Of these, 89 (31.2%) were honeybees (*Apis melifera*), and 49 (17.2%) were bumblebees (*Bombus* spp.), representing five species (Appendix B). The remaining non-apoid visitors (28.2%) included ten other hymenopteran specimens (2.5%), 47 beetles (11.8%), and 47 flies (11.8%). Eighty-four honeybees and 41 bumblebees were selected for pollen load analysis in this study, representing 94.4% and 83.6% of the total number of individuals collected for each respective taxon.

Pollen Load Sequences

*Sequence recoverability*

Fifty-two (41.6%) *rbcL* sequences were recovered from the 125 pollen loads analyzed (Table 2). Of these, 45 were full length and bi-directional. Twenty-five (48.1%) of these sequences were from honeybees, and 27 (51.9%) were from bumblebees. The mean

overall recovery of sequences from pollen loads was 41.6%, with a much higher recovery rate from bumblebees (65.8%) than from honeybees (29.7%; $X^2 = 5.67$, df = 1, P = 0.02; Appendix C).

*Validity and accuracy*

Twenty pollen loads (38.5%) yielded homozygous sequences, indicating a single pollen type (monospecific), and all but one were identifiable to the plant sequence library by ≥ 99.5% identical sites (Fig. 2). Homozygous sequences also contained no more than three ambiguous bases ( $\bar{x} = 0.2$), compared to heterozygous sequences, which possessed between 17 and 115 ( $\bar{x} = 55$). For heterozygous (or mixed pollen load) sequences, the proportion of ambiguous bases located at sites informative for the local flora was 93.4% on average, with a range between 69.4% and 100% (Appendix C).

Pollen load sequences generally displayed expected results, when compared to microscopy analysis (Fig. 3). Only ten pollen loads (19.2%) showed discrepancies between molecular and microscopy results. In eight of these, DNA sequencing predicted a homospecific pollen load (single pollen type), whereas microscopy revealed a mixed pollen load. In two cases, molecular analysis revealed species diversity that was not observed with microscope analysis of pollen loads. In addition, microscopy and molecular analysis never indicated incongruent homospecific identities.

Heterospecific Visitation

According to both microscopy and molecular analysis, all plant species received heterospecific visitation, as indicated by both heterospecific and mixed pollen loads (Fig. 4, Table 3). The majority of visits to *H. piloselloides* (66.7%), *T. sebifera* (90.9%), and *V.*

*brasiliensis* (90.0%) were heterospecific, and *O. rubra* and *S. floridana* received only heterospecific visits. However, only 47.1% of *T. repens* visitors carried heterospecific pollen loads. When considering honeybees alone, only 25.0% of *T. repens* visits indicated heterospecific visitation, and *T. repens* was the only plant species on which bumblebees exhibited exclusive conspecific visitation. Although higher rates of heterospecific visitation were observed to native plant species, there were no statistically significant differences between rates of heterospecific visitation to native and non-native species (1-way ANOVA, $F_{1,4} = 2.78$, $P = 0.171$; Fig. 5).

Heterospecific visitation was also found for most individuals of both honeybees and bumblebees. Sixty-four percent of honeybees and 88.9% of bumblebees were found to carry heterospecific or mixed pollen loads (Fig. 5). Most visitors (61.5%) carried mixed pollen loads, and the remaining 15.4% were found to carry pollen from only a single heterospecific plant species. Honeybees carried three heterospecific, 13 mixed and nine conspecific pollen loads, while bumblebees carried five, nineteen and three (Table 3). However, a 1-way ANOVA revealed no significant difference between the groups ($F_{1,9} = 3.46$, $P = 0.096$; Fig. 5). It was also determined that there was no statistical interaction between pollinator type and plant species nativity.

## DISCUSSION

The primary goal of this study was to assess the utility of plant DNA barcodes for research concerning pollination mutualisms. My results indicate: 1) that plant DNA barcode sequences can be recovered from pollen loads; 2) that when visitors carry monospecific pollen loads, species-level identification of pollen loads can be determined

with high accuracy; and 3) that levels of heterospecific visitation and potential IPT can be assessed with these techniques. To the best of my knowledge, this is the first study to utilize plant DNA barcodes for the molecular analysis of pollen loads.

Sequence recovery

Overall sequence recovery (42%) was higher than that previously reported for an analysis of herbivore gut contents (35%; Navarro *et al.* (2010). Differences in rates of recovery could partially be due to the fact that Navarro *et al.* (2010) attempted to amplify a plant region with DNA extracted from whole insect bodies, while I extracted DNA from pollen loads alone. Although extracting DNA from the insects and their associated plant material is attractive for efficiency reasons, the approach has limits as well. In this study, pollen loads were removed from the external surface of bees for extraction instead of macerating whole insects because 1) pollen present in the digestive tract may not indicate that conspecific pollen was ever carried on the outside of the insect, where it would be available for transfer to con- or heterospecific stigmas; 2) the abundance of insect DNA could hinder the recovery of pollen or plant DNA, due to saturation of DNA binding surfaces associated with the extraction process; 3) whole insect maceration would be difficult for large insects in a high-throughput setting and avoiding this technique will ensure a more consistent analysis across all pollinator types; and 4) whole insect maceration prevents accession of insect vouchers.

Furthermore, the size of the organism being studied may be correlated with the amount of plant tissue available within or on the external surface of specimens, affecting the likelihood of recovering sufficient amounts of plant DNA. Navarro *et al.* (2010)

examined weevils, a group of beetles noted for their diminutive size (≤6mm). When considering the size of their specimens, the overall sequence recovery rate of 35% is somewhat impressive. In this study, pollen loads from *Bombus* spp. (~14mm) were most likely to yield high quality sequences (65.8%). Pollen loads from honeybees (~11mm), which are shorter in length and much less robust, yielded sequences only 29.7% of the time. Thus, the potential for recovering plant barcode sequences from herbivores or floral visitors is likely greater in larger insects.

The use of the *rbcL* barcoding region may also have facilitated the higher recovery rates seen here, as most "barcoding" approaches to plant diet analysis have utilized the *trnL* intron (Valentini *et al.* 2009, Jurado-Rivera *et al.* 2009, Pegard *et al.* 2009, Soininen *et al.* 2009, Navarro *et al.* 2010; but see Bradley *et al.* 2007, Matheson *et al.* 2008). Although the *trnL* intron has demonstrated high species-level discrimination and is thought to be useful for PCR amplification from degraded DNA (Kress & Erickson 2007, Taberlet *et al.* 2007), the *rbcL* region has consistently exhibited one of the highest rates of PCR success among putative barcoding regions, second only to *trnH-psbA* (Kress *et al.* 2005, Kress & Erickson 2007, Fazekas *et al.* 2008). In addition, because pollen load analysis should not usually involve amplification from degraded DNA (except perhaps for museum specimens), the *rbcL* region currently provides the most potential for future barcoding work involving pollen loads.

Species Identification of Monospecific Pollen Loads

The construction of a local *rbcL* sequence library allowed much greater species-level resolution for pollen loads than has been obtained from other analyses. In insect

herbivore gut content studies, Jurado-Rivera *et al.* (2009) and Navarro *et al.* (2010) compared their recovered *trnL* sequences to the GenBank dataset and obtained highest matches with sequence divergences from 0-5.5% and 0-10.7%, allowing identification to the genus-level for only 51% and 56% of sequences, respectively. Similarly, Pegard *et al.* (2009) and Soininen *et al.* (2009), using *trnL* for the analyses of mammal stomach contents and feces, were only able to identify 51.1% and 75% of species sequences to the genus-level. When compared to a local sequence library, however, *rbcL* sequences from monospecific pollen loads (this study) yielded sequences divergences of ≤0.5%, except for one sequence. Likewise, Valentini *et al.* (2009) generated a local *trnL* library and were able to identify 75% of samples to the species-level. In general, until barcode libraries mature, the generation of local sequence databases is essential to reaching species-level discrimination for samples of interest (Jurado-Rivera *et al.* 2009). My study contributes directly to this effort and highlights the importance of developing local barcode libraries for ecological barcoding.

Heterospecific Visitation

The high rates of heterospecific visitation observed here are similar to those observed by other studies. For example, Bartomeus *et al.* (2008) reported that 77% of visitors to five native species in Spain carried pollen from a co-flowering invasive species. Furthermore, in a meta-analysis of sixteen studies, Morales & Traveset (2008) reported that pollinator sharing by plant species ranged from 5 to 100% and that plant-to-plant transitions by visitors varied between 9 and 65%, both indicating high potential for IPT. In the few studies that have reported actual IPT, researchers have observed rates ranging from 4 to

50% (McLernon *et al.* 1996). However, actual rates of heterospecific visitation and IPT, though, vary significantly depending on many factors, including the plant species examined, other plant species present, and the species and abundances of visitors present. The impact of IPT from invasive plants to native species is also highly variable, and the consequence of non-native invasion must be assessed on a case-by-case basis.

The contributions of individual visitor species may also depend on various conditions. For example, although bumblebee species are generally thought to be among the most constant of pollinators (Morales & Traveset 2008), my study revealed a slightly higher rate of constancy by honeybees than *Bombus* spp. This finding supports evidence that bumblebees have the ability to remain constant on several species simultaneously without experiencing reductions in foraging efficiency (Raine & Chittka 2007). This type of multi-species foraging is thought to be especially likely to occur when patches of co-flowering species exist in close proximity (Raine & Chittka 2007), as was certainly case in the collection area for this study.

Heterospecific Pollen Loads

The main limitation of pollen load barcoding is that when pollen loads are mixed, standard Sanger sequencing methods are unable to resolve the identity of the constituent species. I explored the use of alternative base-calling techniques to identify the major component of heterozygous sequences. After base-calling all heterozygous positions as the most dominant nucleotide, I was able to improve the average BLASTn percent identity of heterozygous sequences from 90.7% to 98.4%; however, only 14 (43.8%) of the 32 heterozygous sequences scored BLASTn hits ≥ 99.5% and minor pollen species

were not identified. While this approach does provide some insight into the composition of pollen loads, the level of neglected information is not ideal, especially for studies with small sample sizes or when rare plant or visitor species are involved (Alarcon 2009).

Several methods, though, can be used to resolve environmental samples. Vector cloning is one method by which to isolate individual sequences from complex mixtures (Jurado-Rivera *et al.* 2009), but the method can be time consuming and expensive. Analysis of terminal restriction fragment length polymorphisms (T-RFLP) has also been used to gain insight into complex samples. It is commonly used to assess the microbial diversity of soils (Kirk *et al.* 2004) and has also been used to assess the diversity of bacterial symbionts in termite guts (Trakulnaleamsai *et al.* 2004), to identify bacterial pathogens (Nilsson & Stom 2002), and to determine the source of bloodmeals in mosquitoes (Meece *et al.* 2005). However, because multiple species may produce similar fragment lengths and because even single nucleotide polymorphisms can drastically change a specimen's fragment profile, T-RFLP fails to provide unambiguous species-level identification or to account for intraspecific variation.

Alternatively, pyrosequencing has been used in the identification of herbivore diets and is capable of generating thousands of sequences per mixed sample (Pegard *et al.* 2009, Soininen *et al.* 2009). This type of analysis, which is now being provided by many sequencing facilities, requires little if any additional equipment or skills other than those needed for standard PCR amplification, and although previously cost-prohibitive, the technology is becoming increasingly more affordable (Pegard *et al.* 2009). New advances in pyrosequencing technology are also permitting the sequencing of fragments longer than has previously been possible (454 Life Sciences, www.454.com). As such,

pyrosequencing appears to be the emerging method of choice for ecological barcoding and for molecular analysis of pollen loads (Mike Wilkinson, personal communication).

Implications

Overall, DNA barcoding of pollen loads offers a viable alternative to other currently available techniques for determining plant-pollinator interactions. Most pollination networks, for example, have been based on observation of visitation (Alarcon 2009); however, separate observations of visitation to heterospecific plant species does not necessarily denote interspecific visitation. In addition, even confirmed interspecific visitation does not equate to IPT, and few studies address this by testing for the presence of pollen on visitors or stigmas. Reliance on observation also tends to lead to assumptions of specialization by rare plants or visitors due to an overall lack of data (Alarcon 2009). DNA barcoding eliminates both of these problems by indicating visitation only to plant species where a visitor has obtained significant quantities of plant tissue(s) and by potentially providing information for visits in addition to those observed during visitor collection. Morphological analysis of pollen loads on stigmas may be useful as a means of assessing IPT, but it does not provide any information about the visitor(s), and the pollen is not always easily identifiable (Zhou *et al.* 2007). The barcoding of pollen present on stigmatic surfaces still remains to be explored, and as such, a combination of pollen load barcoding and morphological stigma analysis may currently represent the ideal approach for future IPT studies. Ultimately, pollen barcoding will be especially useful in studies of plant hybridization or pollinator competition among closely related plant species, where pollen types are otherwise difficult or impossible to distinguish. As

pyrosequencing improves and becomes more affordable, and as DNA barcode sequence databases mature, pollen barcoding will also become an increasingly attractive alternative.

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**Table 1.** Distribution of floral visitors collected from a disturbed forest edge on the Columbus State University campus, Columbus Georgia (N 32.4996° W 84.9367°) between May 11 and June 1, 2009.

| **Taxa** | | | **Number Collected** |
|---|---|---|---|
| Coleoptera | | | 47 |
| Diptera | | | 47 |
| Hymenoptera | | | 295 |
| | bees | | 285 |
| | | *Apis mellifera* | 89 |
| | | *Bombus* spp. | 49 |
| | | other | 147 |
| | non-apoid | | 10 |
| TOTAL | | | 389 |

**Table 2.** Polymerase chain reaction (PCR) and sequencing success of the plant barcode marker *rbcL* for DNA isolated from the pollen loads of two bee taxa; results for each visitor type and combined.

| | Plant species | DNA Extractions | Successful PCR Amplification | | Successful Sanger Sequencing | | Overall Success |
|---|---|---|---|---|---|---|---|
| *Apis mellifera* | *Hieracium piloselloides* | 9 | 1 | 11% | 1 | 100% | 11% |
| | *Oxalis rubra* | 2 | 0 | 0% | - | - | 0% |
| | *Stachys floridana* | 16 | 3 | 19% | 1 | 33% | 6% |
| | *Triadica sebifera* | 22 | 13 | 59% | 9 | 69% | 41% |
| | *Trifolium repens* | 12 | 10 | 83% | 8 | 80% | 67% |
| | *Verbena brasiliensis* | 23 | 14 | 61% | 6 | 43% | 26% |
| | TOTAL | 84 | 41 | 49% | 25 | 61% | 30% |
| *Bombus* spp. | *Hieracium piloselloides* | 6 | 2 | 33% | 2 | 100% | 33% |
| | *Oxalis rubra* | 3 | 2 | 67% | 2 | 100% | 67% |
| | *Stachys floridana* | 10 | 8 | 80% | 8 | 100% | 80% |
| | *Triadica sebifera* | 2 | 2 | 100% | 2 | 100% | 100% |
| | *Trifolium repens* | 9 | 9 | 100% | 9 | 100% | 100% |
| | *Verbena brasiliensis* | 11 | 4 | 36% | 4 | 100% | 36% |
| | TOTAL | 41 | 27 | 66% | 27 | 100% | 66% |
| All Visitors | *Hieracium piloselloides* | 15 | 3 | 20% | 3 | 100% | 20% |
| | *Oxalis rubra* | 5 | 2 | 40% | 2 | 100% | 40% |
| | *Stachys floridana* | 26 | 11 | 42% | 9 | 82% | 35% |
| | *Triadica sebifera* | 24 | 15 | 63% | 11 | 73% | 46% |
| | *Trifolium repens* | 21 | 19 | 90% | 17 | 89% | 81% |
| | *Verbena brasiliensis* | 34 | 18 | 53% | 10 | 56% | 29% |
| | Grand Total | 125 | 68 | 54% | 52 | 76% | 42% |

**Table 3.** Percent of pollen load sequences indicating conspecific, heterospecific or mixed pollen loads for six plant species; results for each visitor type and combined.

| | | | Homozygous Sequences | | | | Heterozygous Sequences | |
|---|---|---|---|---|---|---|---|---|
| | | | Conspecific Pollen Load | | Heterospecific Pollen Load | | Mixed Pollen Load | |
| | Plant Species | Number of Sequences | | | | | | |
| *Apis mellifera* | *Hieracium piloselloides* | 1 | 1 | 100% | 0 | 0% | 0 | 0% |
| | *Oxalis rubra* | 0 | - | - | - | - | - | - |
| | *Stachys floridana* | 1 | 0 | 0% | 0 | 0% | 1 | 100% |
| | *Triadica sebifera* | 9 | 1 | 11% | 0 | 0% | 8 | 89% |
| | *Trifolium repens* | 8 | 6 | 75% | 1 | 13% | 1 | 13% |
| | *Verbena brasiliensis* | 6 | 1 | 17% | 2 | 33% | 3 | 50% |
| | TOTAL | 25 | 9 | 36% | 3 | 12% | 13 | 52% |
| *Bombus spp.* | *Hieracium piloselloides* | 2 | 0 | 0% | 0 | 0% | 2 | 100% |
| | *Oxalis rubra* | 2 | 0 | 0% | 0 | 0% | 2 | 100% |
| | *Stachys floridana* | 8 | 0 | 0% | 2 | 25% | 6 | 75% |
| | *Triadica sebifera* | 2 | 0 | 0% | 0 | 0% | 2 | 100% |
| | *Trifolium repens* | 9 | 3 | 33% | 1 | 11% | 5 | 56% |
| | *Verbena brasiliensis* | 4 | 0 | 0% | 2 | 50% | 2 | 50% |
| | TOTAL | 27 | 3 | 11% | 5 | 19% | 19 | 70% |
| All Visitors | *Hieracium piloselloides* | 3 | 1 | 33% | 0 | 0% | 2 | 67% |
| | *Oxalis rubra* | 2 | 0 | - | 0 | - | 2 | 100% |
| | *Stachys floridana* | 9 | 0 | 0% | 2 | 22% | 7 | 78% |
| | *Triadica sebifera* | 11 | 1 | 9% | 0 | 0% | 10 | 91% |
| | *Trifolium repens* | 17 | 9 | 53% | 2 | 12% | 6 | 35% |
| | *Verbena brasiliensis* | 10 | 1 | 10% | 4 | 40% | 5 | 50% |
| | Grand Total | 52 | 12 | 23% | 8 | 15% | 32 | 62% |

**Figure 1.** Selection of *rbcL* chromatographs (bp 110-114) for PCR amplicon produced by amplifying mixtures of plant DNA (*Stachys florida* and *Oxalis rubra*) at various ratios. Percentages indicate proportion of PCR template composed of *S. florida* DNA.

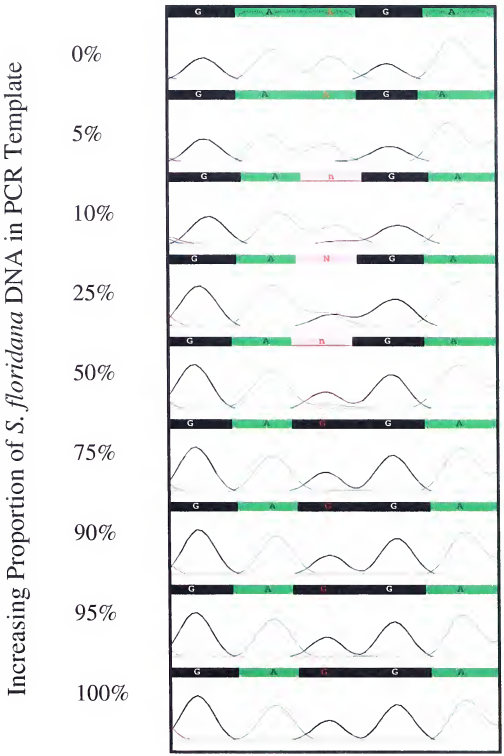


**Figure 2.** Plant sources of sequences recovered from honeybee and bumblebee pollen loads. Numbers in parentheses indicate number of visitors collected from each individual plant species.

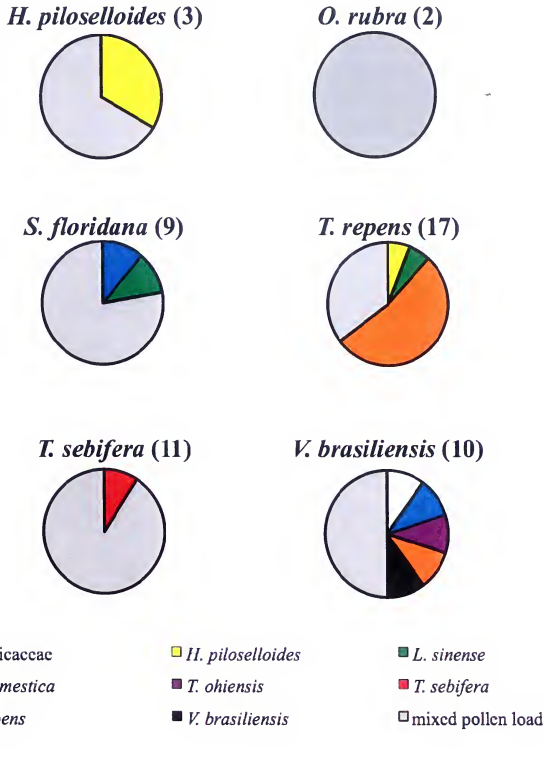


**Figure 3.** Pollen slides and corresponding sequence chromatographs generated from pollen loads of *Trifolium repens* visitors. A) *T. repens*, plant reference, CSU016-1; B) *T. repens*, conspecific visitation, A559; C) Asteraceae (*Hieracium piloselloides* or *Sonchus asper*), identifiable heterospecific visitation, A298; D) mixed pollen load, unidentifiable heterospecific visitation, A616.

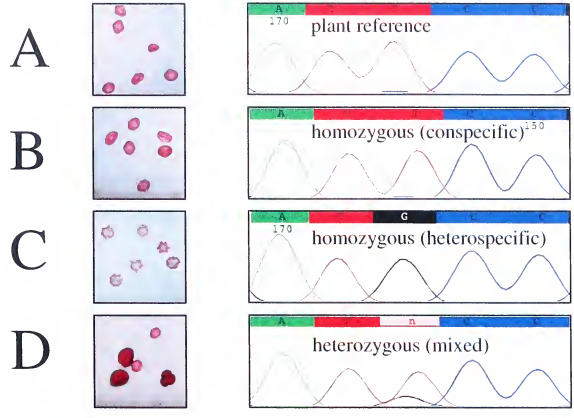


**Figure 4.** Rates of heterospecific visitation expressed as percentage of pollen load sequences yielding identifiable heterospecific or mixed pollen loads. A) Total heterospecific visitation experienced by *Hieracium piloselloides*, *Oxalis rubra*, *Stachys floridana*, *Triadica sebifera*, *Trifolium repens* and *Verbena brasiliensis*; B) heterospecific visitation by honeybees; C) heterospecific visitation by bumble bees. Numbers above bars indicate number of pollen load sequences per plant species.

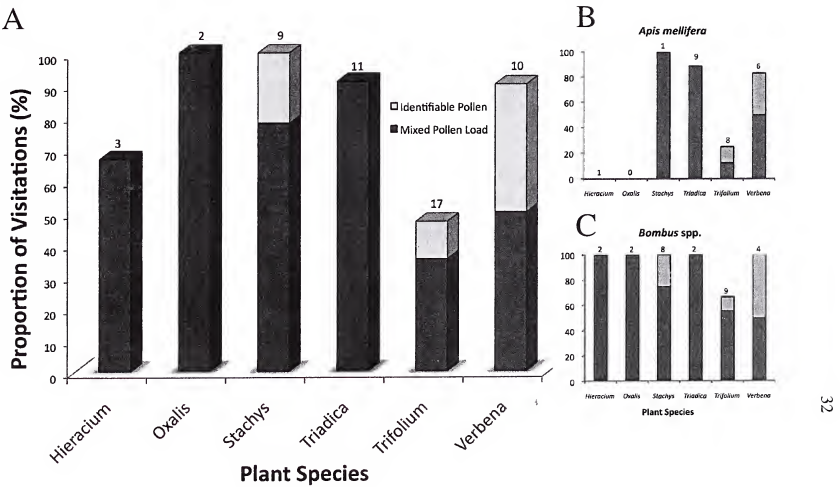
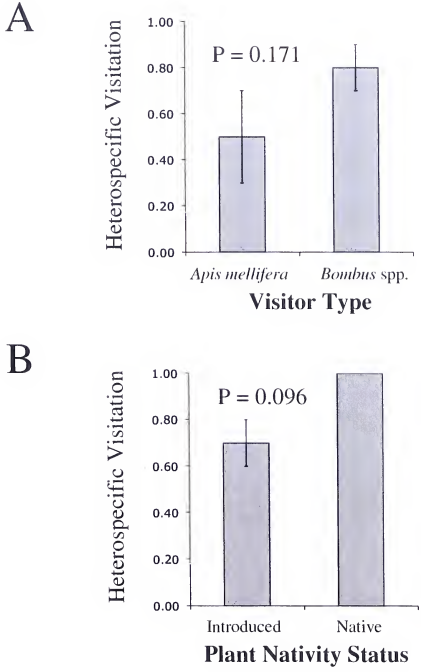


**Figure 5.** Mean (±1 S.E.) proportions of heterospecific visitation. A) No significant difference was found between rates of heterospecific vistation by honeybee (n = 25) and bumblebee (n = 27) visitors (1-way ANOVA, $F_{1,4} = 2.78$, P = 0.171); B) Difference between heterospecific visitation received by native (n = 2) and introduced (n = 4) plant species was also found to be non-significant (1-way ANOVA, $F_{1,9} = 3.46$, P = 0.096).



# APPENDIX A

## Plant DNA barcode vouchers

| Plant ID | Herb ID # | Taxa* | Family | Nativity** | Sequences | | | | | BOLD Process # | GenBank Accession # |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | F/R | F | R | bp | # Ns | | |
| CSU001 | 1001 | *Glandularia pulchella* (Sweet) Troncoso | Verbenaceae | I | 3x | 2x | 1x | 607 | - | CSU017-10 | HQ644044 |
| CSU003 | 1003 | *Hieracium piloselloides* Vill. | Asteraceae | I | 3x | 2x | 1x | 607 | - | CSU018-10 | HQ644046 |
| CSU004 | 1004 | *Tradescantia ohiensis* Raf. | Commelinaceae | N | 3x | 2x | 1x | 607 | - | CSU019-10 | HQ644075 |
| CSU005 | 1005 | *Stachys floridana* Shuttlw. ex Benth. | Lamiaceae | N | 3x | 2x | 1x | 607 | - | CSU001-10 | HQ644074 |
| CSU006 | 1006 | *Vicia grandiflora* Scop. | Fabaceae | I | 3x | 2x | 1x | 607 | - | CSU020-10 | HQ644081 |
| CSU007 | 1007 | *Ligustrum sinense* Lour. | Oleaceae | I | 3x | 2x | 1x | 607 | - | CSU021-10 | HQ644051 |
| CSU008 | 1008 | *Verbena brasiliensis* Vell. | Verbenaceae | I | 3x | 2x | 1x | 607 | - | CSU022-10 | HQ644080 |
| CSU009 | 1009 | *Lonicera japonica* Thunb. | Caprifoliaceae | I | 3x | 2x | 1x | 607 | - | CSU023-10 | HQ644053 |
| CSU010 | 1010 | *Sisyrinchium rosulatum* E.P. Bicknell | Iridaceae | N | 3x | 2x | 1x | 607 | - | CSU024-10 | HQ644070 |
| CSU011 | 1011 | *Oenothera laciniata* Hill | Onagraceae | N | 3x | 2x | 1x | 607 | - | CSU002-10 | HQ644057 |
| CSU012 | 1012 | *Oxalis rubra* A. St.-Hil. | Oxalidaceae | N | 3x | 2x | 1x | 607 | - | CSU025-10 | HQ644060 |
| CSU013 | 1013 | *Plantago lanceolata* L. | Plantaginaceae | I | 3x | 2x | 1x | 607 | - | CSU026-10 | HQ644063 |

continued

| Plant ID | Herb ID # | Taxa* | Family | Nativity** | Sequences | | | | | BOLD Process # | GenBank Accession # |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CSU015 | 1015 | *Krigia virginica* (L.) Willd. | Asteraceae | N | 3x | 2x | 1x | 607 | - | CSU027-10 | HQ644050 |
| CSU016 | 1016 | *Mazus pumilus* (Burm. f.) Steenis | Scrophulariaceae | I | 3x | 2x | 1x | 607 | - | CSU028-10 | HQ644054 |
| CSU016-1 | 1058 | *Trifolium repens* L. | Fabaceae | I | 3x | 2x | 1x | 607 | - | CSU029-10 | HQ644078 |
| CSU017 | 1017 | *Oxalis dillenii* Jacq. | Oxalidaceae | N | 3x | 2x | 1x | 607 | - | CSU003-10 | HQ644059 |
| CSU017-1 | 1059 | *Oenothera speciosa* Nutt. | Onagraceae | N | 3x | 2x | 1x | 607 | - | CSU030-10 | HQ644058 |
| CSU018 | 1018 | *Packera anonyma* (Alph. Wood) W.A. Weber & A. Löve | Asteraceae | N | 3x | 2x | 1x | 607 | - | CSU004-10 | HQ644061 |
| CSU019 | 1019 | *Wahlenbergia marginata* (Thunb.) A. DC. | Campanulaceae | I | 3x | 2x | 1x | 607 | - | CSU031-10 | HQ644084 |
| CSU020 | 1020 | *Rumex* L. | Polygonaceae | - | 3x | 2x | 1x | 607 | - | CSU005-10 | HQ644065 |
| CSU021 | 1021 | *Allium canadense* L. | Liliaceae | N | 3x | 2x | 1x | 607 | - | CSU032-10 | HQ644038 |
| CSU022 | 1022 | *Solanum carolinense* L. | Solanaceae | I | 9x | 6x | 3x | 607 | - | CSU033-10 | HQ644071 |
| CSU024 | 1024 | *Hemerocallis fulva* (L.) L. | Liliaceae | I | 3x | 2x | 1x | 607 | 1 | CSU034-10 | HQ644045 |
| CSU024-1 | 1060 | *Sonchus asper* (L.) Hill | Asteraceae | I | 3x | 2x | 1x | 607 | - | CSU035-10 | HQ644073 |
| CSU025 | 1025 | *Alstroemeria psittacina* Lehm., nom. inq. | Liliaceae | I | 3x | 2x | 1x | 607 | - | CSU036-10 | HQ644039 |
| CSU025-1 | 1061 | *Hypochaeris brasiliensis* (Less.) Benth. & Hook. ex Griseb. | Asteraceae | I | 3x | 2x | 1x | 607 | - | CSU037-10 | HQ644047 |

continued

| Plant ID | Herb ID # | Taxa* | Family | Nativity** | Sequences | | | | | BOLD Process # | GenBank Accession # |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CSU026 | 1026 | *Triodanis perfoliata* (L.) Nieuwl. | Campanulaceae | N | 3x | 2x | 1x | 607 | - | CSU038-10 | HQ644079 |
| CSU027 | 1027 | *Scutellaria parvula* Michx. | Lamiaceae | N | 3x | 2x | 1x | 607 | - | CSU039-10 | HQ644068 |
| CSU028 | 1028 | *Liriope spicata* (Thunb.) Lour. | Ruscaceae | I | 3x | 2x | 1x | 607 | - | CSU040-10 | HQ644052 |
| CSU029 | 1029 | *Pyrrhopappus carolinianus* (Walter) DC. | Asteraceae | N | 3x | 2x | 1x | 607 | - | CSU041-10 | HQ644064 |
| CSU031 | 1031 | *Sambucus nigra* L. ssp. *canadensis* (L.) R. Bolli | Caprifoliaceae | N | 3x | 2x | 1x | 607 | - | CSU042-10 | HQ644067 |
| CSU032 | 1032 | *Gamochaeta purpurea* (L.) Cabrera | Asteraceae | N | 3x | 2x | 1x | 607 | - | CSU006-10 | HQ644042 |
| CSU036 | 1036 | *Petunia* Juss. | Solanaceae | I | 3x | 2x | 1x | 607 | - | CSU007-10 | HQ644062 |
| CSU037 | 1037 | *Vitis aestivalis* Michx. | Vitaceae | N | 9x | 6x | 3x | 607 | 3 | CSU008-10 | HQ644082 |
| CSU038 | 1038 | *Solanum physalifolium* Rusby | Solanaceae | I | 3x | 2x | 1x | 607 | - | CSU009-10 | HQ644072 |
| CSU039 | 1039 | *Rumex crispus* L. | Polygonaceae | I | 3x | 2x | 1x | 607 | - | CSU010-10 | HQ644066 |
| CSU040 | 1040 | *Geranium carolinianum* L. | Geraniaceae | N | 2x | 1x | 1x | 607 | - | CSU011-10 | HQ644043 |
| CSU041 | 1041 | *Trifolium arvense* L. | Fabaceae | I | 3x | 2x | 1x | 607 | - | CSU043-10 | HQ644077 |
| CSU044 | 1044 | *Abelia ×grandiflora* (Rovelli ex André) Rehder [*chinensis* × *uniflora*] | Caprifoliaceae | I | 1x | 1x | - | 584 | - | CSU044-10 | HQ644037 |
| CSU045 | 1045 | *Jasminum* L. | Oleaceae | I | 1x | 1x | - | 587 | - | CSU012-10 | HQ644049 |

continued

| Plant ID | Herb ID # | Taxa* | Family | Nativity** | Sequences | | | | | BOLD Process # | GenBank Accession # |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CSU046 | 1046 | *Ipomoea pandurata* (L.) G. Mey. | Convolvulaceae | N | 3x | 2x | 1x | 607 | 1 | CSU045-10 | HQ644048 |
| CSU047 | 1047 | *Nandina domestica* Thunb. | Berberidaceae | I | 3x | 2x | 1x | 607 | - | CSU046-10 | HQ644056 |
| CSU048 | 1048 | *Sherardia arvensis* L. | Rubiaceae | I | 3x | 2x | 1x | 607 | - | CSU047-10 | HQ644069 |
| CSU050 | 1050 | *Diodia virginiana* L. | Rubiaceae | N | 4x | 2x | 2x | 607 | - | CSU048-10 | HQ644041 |
| CSU052 | 1052 | *Medicago lupulina* L. | Fabaceae | I | 4x | 3x | 1x | 607 | - | CSU049-10 | HQ644055 |
| CSU053 | 1053 | *Youngia japonica* (L.) DC. | Asteraceae | I | 3x | 2x | 1x | 607 | - | CSU013-10 | HQ644085 |
| CSU055 | 1055 | *Vitis rotundifolia* Michx. | Vitaceae | N | 3x | 2x | 1x | 607 | - | CSU014-10 | HQ644083 |
| CSU056 | 1056 | *Triadica sebifera* (L.) Small | Euphorbiaceae | I | 4x | 3x | 1x | 607 | - | CSU015-10 | HQ644076 |
| CSU057 | 1057 | *Ampelopsis arborea* (L.) Koehne | Vitaceae | N | 3x | 2x | 1x | 607 | - | CSU016-10 | HQ644040 |

* most current names and authorities from USDA Plants Database, http://plants.usda.gov/

** nativity to the lower 48 U.S. states from USDA Plants Database; N = native; I = introduced.

# APPENDIX B

## Bee voucher information

| Insect ID | Species * | Nativity ** | Forage Plant | Pollen Load |
|---|---|---|---|---|
| A301 | *Apis mellifera* Linnaeus, 1758 | I | *Hieracium piloselloides* | con |
| A123 | *Apis mellifera* Linnaeus, 1758 | I | *Stachys floridana* | het (mix) |
| A572 | *Apis mellifera* Linnaeus, 1758 | I | *Triadica sebifera* | het (mix) |
| A573 | *Apis mellifera* Linnaeus, 1758 | I | *Triadica sebifera* | het (mix) |
| A574 | *Apis mellifera* Linnaeus, 1758 | I | *Triadica sebifera* | het (mix) |
| A586 | *Apis mellifera* Linnaeus, 1758 | I | *Triadica sebifera* | het (mix) |
| A587 | *Apis mellifera* Linnaeus, 1758 | I | *Triadica sebifera* | con |
| A589 | *Apis mellifera* Linnaeus, 1758 | I | *Triadica sebifera* | het (mix) |
| A592 | *Apis mellifera* Linnaeus, 1758 | I | *Triadica sebifera* | het (mix) |
| A599 | *Apis mellifera* Linnaeus, 1758 | I | *Triadica sebifera* | het (mix) |
| A628 | *Apis mellifera* Linnaeus, 1758 | I | *Triadica sebifera* | het (mix) |
| A227 | *Apis mellifera* Linnaeus, 1758 | I | *Trifolium repens* | het (mix) |
| A298 | *Apis mellifera* Linnaeus, 1758 | I | *Trifolium repens* | het |
| A558 | *Apis mellifera* Linnaeus, 1758 | I | *Trifolium repens* | con |
| A559 | *Apis mellifera* Linnaeus, 1758 | I | *Trifolium repens* | con |
| A561 | *Apis mellifera* Linnaeus, 1758 | I | *Trifolium repens* | con |
| A593 | *Apis mellifera* Linnaeus, 1758 | I | *Trifolium repens* | con |
| A605 | *Apis mellifera* Linnaeus, 1758 | I | *Trifolium repens* | con |
| A606 | *Apis mellifera* Linnaeus, 1758 | I | *Trifolium repens* | con |

continued

| Insect ID | Species * | Nativity ** | Forage Plant | Pollen Load |
|---|---|---|---|---|
| A245 | *Apis mellifera* Linnaeus, 1758 | I | *Verbena brasiliensis* | het (mix) |
| A275 | *Apis mellifera* Linnaeus, 1758 | I | *Verbena brasiliensis* | het |
| A461 | *Apis mellifera* Linnaeus, 1758 | I | *Verbena brasiliensis* | het |
| A486 | *Apis mellifera* Linnaeus, 1758 | I | *Verbena brasiliensis* | con |
| A492 | *Apis mellifera* Linnaeus, 1758 | I | *Verbena brasiliensis* | het (mix) |
| A497 | *Apis mellifera* Linnaeus, 1758 | I | *Verbena brasiliensis* | het (mix) |
| A119 | *Bombus bimaculatus* Cresson, 1863 | N | *Stachys floridana* | het (mix) |
| A133 | *Bombus bimaculatus* Cresson, 1863 | N | *Stachys floridana* | het (mix) |
| A148 | *Bombus bimaculatus* Cresson, 1863 | N | *Stachys floridana* | het (mix) |
| A310 | *Bombus bimaculatus* Cresson, 1863 | N | *Stachys floridana* | het |
| A562 | *Bombus bimaculatus* Cresson, 1863 | N | *Trifolium repens* | con |
| A595 | *Bombus bimaculatus* Cresson, 1863 | N | *Trifolium repens* | con |
| A616 | *Bombus bimaculatus* Cresson, 1863 | N | *Trifolium repens* | het (mix) |
| A617 | *Bombus bimaculatus* Cresson, 1863 | N | *Trifolium repens* | het (mix) |
| A493 | *Bombus bimaculatus* Cresson, 1863 | N | *Verbena brasiliensis* | het |
| A612 | *Bombus bimaculatus* Cresson, 1863 | N | *Verbena brasiliensis* | het (mix) |
| A215 | *Bombus fraternus* (Smith, 1863) | N | *Hieracium piloselloides* | het (mix) |
| A137 | *Bombus fraternus* (Smith, 1863) | N | *Oxalis rubra* | het (mix) |
| A117 | *Bombus griseocollis* (DeGeer, 1773) | N | *Stachys floridana* | het |
| A578 | *Bombus griseocollis* (DeGeer, 1773) | N | *Triadica sebifera* | het (mix) |
| A579 | *Bombus griseocollis* (DeGeer, 1773) | N | *Triadica sebifera* | het (mix) |
| A548 | *Bombus impatiens* Cresson, 1863 | N | *Hieracium piloselloides* | het (mix) |

continued

| Insect ID | Species * | Nativity ** | Forage Plant | Pollen Load |
|---|---|---|---|---|
| A134 | *Bombus impatiens* Cresson, 1863 | N | *Oxalis rubra* | het (mix) |
| A311 | *Bombus impatiens* Cresson, 1863 | N | *Stachys floridana* | het (mix) |
| A312 | *Bombus impatiens* Cresson, 1863 | N | *Stachys floridana* | het (mix) |
| A608 | *Bombus impatiens* Cresson, 1863 | N | *Trifolium repens* | het (mix) |
| A614 | *Bombus impatiens* Cresson, 1863 | N | *Trifolium repens* | con |
| A615 | *Bombus impatiens* Cresson, 1863 | N | *Trifolium repens* | het |
| A618 | *Bombus impatiens* Cresson, 1863 | N | *Trifolium repens* | het (mix) |
| A411 | *Bombus impatiens* Cresson, 1863 | N | *Verbena brasiliensis* | het (mix) |
| A429 | *Bombus impatiens* Cresson, 1863 | N | *Verbena brasiliensis* | het |
| A535 | *Bombus impatiens* Cresson, 1863 | N | *Verbena brasiliensis* | het (mix) |
| A418 | *Bombus pensylvanicus* (DeGeer, 1773) | N | *Stachys floridana* | het (mix) |

* current names and authorities from Integrated Taxonomic Information System (ITIS) website, http://www.itis.gov/index.html

** determined from ITIS website; N = native; I = introduced

# APPENDIX C

## Pollen load sequence information

| | | | Sequences | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Insect ID | Bee species | Plant species | F/R | F | R | bp | # Ns | % Ns | #Ns at inform sites | %Ns at inform sites | hom/het sequence | con/het plant sp | Pollen slides |
| A301 | *Apis mellifera* | *Hieracium piloselloides* | 2x | 1x | 1x | 541 | - | - | - | - | hom | con | con |
| A215 | *Bombus fraternus* | *Hieracium piloselloides* | 2x | 1x | 1x | 607 | 54 | 8.90 | 54 | 100.00 | het | het (mix) | het (mix) |
| A548 | *Bombus impatiens* | *Hieracium piloselloides* | 2x | 1x | 1x | 607 | 17 | 2.80 | 12 | 70.59 | het | het (mix) | het (mix) |
| A137 | *Bombus fraternus* | *Oxalis rubra* | 2x | 1x | 1x | 607 | 55 | 9.06 | 54 | 98.18 | het | het (mix) | het (mix) |
| A134 | *Bombus impatiens* | *Oxalis rubra* | 2x | 1x | 1x | 607 | 33 | 5.44 | 33 | 100.00 | het | het (mix) | het (mix) |
| A123 | *Apis mellifera* | *Stachys floridana* | 2x | 1x | 1x | 607 | 64 | 10.54 | 60 | 93.75 | het | het (mix) | het (mix) |
| A119 | *Bombus bimaculatus* | *Stachys floridana* | 2x | 1x | 1x | 607 | 38 | 6.26 | 38 | 100.00 | het | het (mix) | het (mix) |
| A133 | *Bombus bimaculatus* | *Stachys floridana* | 2x | 1x | 1x | 607 | 51 | 8.40 | 51 | 100.00 | het | het (mix) | het (mix) |
| A148 | *Bombus bimaculatus* | *Stachys floridana* | 2x | 1x | 1x | 607 | 36 | 5.93 | 35 | 97.22 | het | het (mix) | het (mix) |
| A310 | *Bombus bimaculatus* | *Stachys floridana* | 2x | 1x | 1x | 607 | - | - | - | - | hom | het | het |
| A117 | *Bombus griseocollis* | *Stachys floridana* | 2x | 1x | 1x | 607 | - | - | - | - | hom | het | het (mix) |
| A311 | *Bombus impatiens* | *Stachys floridana* | 2x | 1x | 1x | 607 | 33 | 5.44 | 33 | 100.00 | het | het (mix) | het (mix) |
| A312 | *Bombus impatiens* | *Stachys floridana* | 2x | 1x | 1x | 607 | 26 | 4.28 | 26 | 100.00 | het | het (mix) | het (mix) |

continued

| | | | Sequences | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Insect ID | Bee species | Plant species | F/R | F | R | bp | # Ns | % Ns | #Ns at inform sites | %Ns at inform sites | hom/het sequence | con/het plant sp | Pollen slides |
| A418 | *Bombus pennsylvanicus* | *Stachys floridana* | 2x | 1x | 1x | 607 | 27 | 4.45 | 27 | 100.00 | het | het (mix) | het (mix) |
| A572 | *Apis mellifera* | *Triadica sebifera* | 1x | 1x | - | 572 | 83 | 14.51 | 58 | 69.88 | het | het (mix) | con |
| A573 | *Apis mellifera* | *Triadica sebifera* | 1x | 1x | - | 573 | 60 | 10.47 | 55 | 91.67 | het | het (mix) | het (mix) |
| A574 | *Apis mellifera* | *Triadica sebifera* | 2x | 1x | 1x | 607 | 53 | 8.73 | 51 | 96.23 | het | het (mix) | het (mix) |
| A586 | *Apis mellifera* | *Triadica sebifera* | 3x | 2x | 1x | 607 | 70 | 11.53 | 68 | 97.14 | het | het (mix) | con |
| A587 | *Apis mellifera* | *Triadica sebifera* | 2x | 1x | 1x | 607 | - | - | - | - | hom | con | con |
| A589 | *Apis mellifera* | *Triadica sebifera* | 2x | 1x | 1x | 607 | 72 | 11.86 | 66 | 91.67 | het | het (mix) | het (mix) |
| A592 | *Apis mellifera* | *Triadica sebifera* | 1x | 1x | - | 604 | 98 | 16.23 | 68 | 69.39 | het | het (mix) | het (mix) |
| A599 | *Apis mellifera* | *Triadica sebifera* | 3x | 2x | 1x | 581 | 47 | 8.09 | 39 | 82.98 | het | het (mix) | het (mix) |
| A628 | *Apis mellifera* | *Triadica sebifera* | 3x | 2x | 1x | 589 | 48 | 8.15 | 46 | 95.83 | het | het (mix) | het (mix) |
| A578 | *Bombus griseocollis* | *Triadica sebifera* | 2x | 1x | 1x | 607 | 48 | 7.91 | 47 | 97.92 | het | het (mix) | het (mix) |
| A579 | *Bombus griseocollis* | *Triadica sebifera* | 2x | 1x | 1x | 607 | 51 | 8.40 | 50 | 98.04 | het | het (mix) | het (mix) |
| A227 | *Apis mellifera* | *Trifolium repens* | 2x | 1x | 1x | 607 | 35 | 5.77 | 35 | 100.00 | het | het (mix) | het (mix) |
| A298 | *Apis mellifera* | *Trifolium repens* | 2x | 1x | 1x | 607 | - | - | - | - | hom | het | het (mix) |
| A558 | *Apis mellifera* | *Trifolium repens* | 2x | 1x | 1x | 607 | - | - | - | - | hom | con | het (mix) |

continued

| | | | Sequences | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Insect ID | Bee species | Plant species | F/R | F | R | bp | # Ns | % Ns | #Ns at inform sites | %Ns at inform sites | hom/het sequence | con/het plant sp | Pollen slides |
| A559 | *Apis mellifera* | *Trifolium repens* | 2x | 1x | 1x | 607 | - | - | - | - | hom | con | con |
| A561 | *Apis mellifera* | *Trifolium repens* | 2x | 1x | 1x | 607 | - | - | - | - | hom | con | con |
| A593 | *Apis mellifera* | *Trifolium repens* | 2x | 1x | 1x | 607 | - | - | - | - | hom | con | con |
| A605 | *Apis mellifera* | *Trifolium repens* | 2x | 1x | 1x | 607 | 1 | 0.16 | - | - | hom | con | con |
| A606 | *Apis mellifera* | *Trifolium repens* | 2x | 1x | 1x | 607 | - | - | - | - | hom | con | het (mix) |
| A562 | *Bombus bimaculatus* | *Trifolium repens* | 2x | 1x | 1x | 607 | - | - | - | - | hom | con | con |
| A595 | *Bombus bimaculatus* | *Trifolium repens* | 2x | 1x | 1x | 607 | - | - | - | - | hom | con | het (mix) |
| A612 | *Bombus bimaculatus* | *Trifolium repens* | 2x | 1x | 1x | 607 | 86 | 14.17 | 64 | 74.42 | het | het (mix) | het (mix) |
| A616 | *Bombus bimaculatus* | *Trifolium repens* | 2x | 1x | 1x | 607 | 50 | 8.24 | 50 | 100.00 | het | het (mix) | het (mix) |
| A617 | *Bombus bimaculatus* | *Trifolium repens* | 2x | 1x | 1x | 607 | 64 | 10.54 | 64 | 100.00 | het | het (mix) | het (mix) |
| A608 | *Bombus impatiens* | *Trifolium repens* | 2x | 1x | 1x | 607 | 77 | 12.69 | 76 | 98.70 | het | het (mix) | het (mix) |
| A614 | *Bombus impatiens* | *Trifolium repens* | 2x | 1x | 1x | 607 | - | - | - | - | hom | con | con |
| A615 | *Bombus impatiens* | *Trifolium repens* | 2x | 1x | 1x | 607 | - | - | - | - | hom | het | het (mix) |
| A618 | *Bombus impatiens* | *Trifolium repens* | 2x | 1x | 1x | 607 | 55 | 9.06 | 54 | 98.18 | het | het (mix) | het (mix) |
| A245 | *Apis mellifera* | *Verbena brasiliensis* | 2x | 1x | 1x | 607 | 58 | 9.56 | 58 | 100.00 | het | het (mix) | het (mix) |

continued

| Insect ID | Bee species | Plant species | Sequences | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | F/R | F | R | bp | # Ns | % Ns | #Ns at inform sites | %Ns at inform sites | hom/het sequence | con/het plant sp | Pollen slides |
| A275 | *Apis mellifera* | *Verbena brasiliensis* | 2x | 1x | 1x | 607 | 1 | 0.16 | - | - | hom | het | het (mix) |
| A461 | *Apis mellifera* | *Verbena brasiliensis* | 2x | 1x | 1x | 607 | - | - | - | - | hom | het | het (mix) |
| A486 | *Apis mellifera* | *Verbena brasiliensis* | 2x | 1x | 1x | 607 | 1 | 0.16 | - | - | hom | con | con |
| A492 | *Apis mellifera* | *Verbena brasiliensis* | 1x | 1x | - | 512 | 115 | 22.46 | 85 | 73.91 | het | het (mix) | het (mix) |
| A497 | *Apis mellifera* | *Verbena brasiliensis* | 2x | 1x | 1x | 607 | 42 | 6.92 | 39 | 92.86 | het | het (mix) | het (mix) |
| A493 | *Bombus bimaculatus* | *Verbena brasiliensis* | 2x | 1x | 1x | 607 | 1 | 0.16 | - | - | hom | het | het |
| A411 | *Bombus impatiens* | *Verbena brasiliensis* | 2x | 1x | 1x | 607 | 71 | 11.70 | 70 | 98.59 | het | het (mix) | het (mix) |
| A429 | *Bombus impatiens* | *Verbena brasiliensis* | 2x | 1x | 1x | 607 | - | - | - | - | hom | het | het |
| A535 | *Bombus impatiens* | *Verbena brasiliensis* | 2x | 1x | 1x | 607 | 43 | 7.08 | 43 | 100.00 | het | het (mix) | het (mix) |